

Attorney Docket No. 5051-458IP

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Hanley-Bowdoin et al.
Filed: August 4, 2003
Serial No.: 10/633,850
For: *Geminivirus Resistant Transgenic Plants*

Art Unit: 1638
Confirmation No.: 5547
Examiner: Li Zheng

VIA FACSIMILE TRANSMISSION to 571-273-8300

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Linda Hanley-Bowdoin, Ph.D.
Pursuant to 37 C.F.R. § 1.132

I, Linda Hanley-Bowdoin, do hereby declare and say as follows:

1. I am a named inventor on US Application No. 10/633,850 (the '850 application) and of the subject matter claimed therein.
2. I have a Ph.D. from Rockefeller University in New York City, NY. I am a William Neal Reynolds Distinguished Professor and a professor in the Department of Molecular and Structural Biochemistry and Department of Genetics at North Carolina State University. I have been conducting and directing research in the area of geminiviruses/host interactions and disease resistance, transcriptional regulation during the plant cell division cycle and plant DNA replication for 20 years and have authored or co-authored more than 40 publications related to this area.
3. The studies described below were carried out in my laboratory at North Carolina State University in Raleigh, North Carolina, under my direction and supervision according to the disclosure set forth in the '850 application. These studies demonstrate that the presently claimed AL1 nucleic acid sequence encoding an Rb binding mutation (SEQ ID NO:109) in combination with another AL1 mutation affecting viral replication can be used to provide stable transgenic plants which are phenotypically normal and have increased resistance to geminivirus infection.
4. The '850 application describes the use of nucleic acid sequences encoding mutant AL1 proteins (Rep) for the production of geminivirus resistant transgenic plants. Geminiviruses replicate via a rolling circle mechanism that is initiated by the viral AL1 (Rep) protein. Several AL1 mutants that oppose viral replication in cultured cells have been identified and are good candidates for conferring disease resistance to transgenic plants. However, it has proven difficult to recover phenotypically normal transgenic lines that stably express AL1, because of the ability of AL1 to modify plant cell cycle and developmental controls through interactions with retinoblastoma (RBR), a host protein that negatively regulates cell division and facilitates

differentiation. However, with the identification of AL1 mutants that cannot bind to RBR as disclosed in the present invention, it became possible to overcome this limitation.

5. The AL1 protein is the only viral protein essential for viral replication in geminiviruses and functions by inducing the synthesis of host replication machinery in infected plants. (Specification, page 14, lines 21-28) All of the known activities of AL1 except for ATP hydrolysis are mediated by overlapping domains in the N-terminal half of the protein, including an oligomerization domain and an Rb binding domain (Specification, page 17, lines 12-14; page 19, line 24 through page 20, line 24; and page 33, lines 29-32). Mutations in the oligomerization domain result in decreased binding of the mutant AL1 protein to wild type AL1 protein but in some cases can also result in impaired Rb binding. (Specification, page 34, lines 2-9). The effect on Rb binding by mutations in the oligomerization domain is believed to be the result of AL1/AL1 interactions that may be a prerequisite for binding of AL1 to Rb. *Id.* Mutations in the Rb binding domain result in decreased binding of the AL1 protein to the plant retinoblastoma. (Specification, page 12). Mutations in the Rb binding domain that are specific for Rb binding, such as TGMV L148 and CbLCV L145, do not result in concomitant loss in AL1 oligomerization activity. (Specification, page 39, lines 23-26).

Using protoplast transfection studies, we have been able to show that mutations in the oligomerization domain cause reduced viral replication. (Specification, Example 5 and page 35, lines 5-11). These are the studies referred to on page 8, first full paragraph, of the Office Action dated December 19, 2006, wherein the Examiner states that the specification does not teach that expression of the oligomerization domain of SEQ ID NO: 109 or of L148 could reduce wild-type replication similar to those oligomerization-domain-fused GST mutants ala6-9 and ala13-14. The Examiner further states that Figure 3 and Tables 1 and 2 do not confirm the ability of the ala5 oligomerization domain (KEE146 mutant) alone to interfere with wild-type replication.

It is important to understand that the ala5, L145 and L148 mutations are not mutations in the oligomerization domain. Since the mutations specific for reduced Rb binding, such as ala5, TGMV L148 and CbLCV L145, do not result in a loss of AL1 oligomerization activity, it is not expected that they alone would cause reduced viral replication. (Specification, page 39, lines 23-26; *See also* Kong et al. (*EMBO J.* 19: 3485-3495 (2000), pp. 3489-3490) (copy enclosed)). As described in the specification and above, it is the combination of mutations in the Rb binding and the oligomerization domains that results in reduced binding. Thus, the reduced replication of the virus in protoplasts as shown in Tables 1 and 2 of the specification is the result of the KEE146 (ala5) mutation in combination with an oligomerization mutation (e.g., ala4).

6. As taught in the specification and described above, the present invention is directed to the development of transgenic plants with increased resistance to geminiviruses. As noted, the use of AL1 mutants that are modified in the DNA cleavage or ATPase domain to produce transgenic plants with resistance to geminiviruses has been problematic, with a lack of stable expression of AL1 in the transgenic plants. (Specification, page 18, lines 7-11). Thus, the present invention is based on our discovery that combining a transdominant negative mutation such as an oligomerization mutation (and/or a mutation in the DNA cleavage and/or the ATPase domain) with a retinoblastoma binding mutation overcomes this difficulty. (Specification, page 16, line 19 through page 17, line 2; and page 18, lines 7-11). Thus, a stable geminivirus resistant

plant produced by the methods described in the '850 application involves the introduction into the plant genome of an AL1 nucleic acid sequence which comprises a trans-dominant mutation and an Rb binding mutation, such as L145 or L148.

7. In order to determine if co-expression of mutant Rep (AL1) and C3 proteins in transgenic plants confers stable geminivirus resistance, we constructed a bicistronic cassette corresponding to TGMV Rep and C3 that carries four mutations in conserved sequences – Rep and C3 transdominant mutations, a pRBR binding mutation in Rep, and a C2 ATG mutation to block expression. Transient replication assays in tobacco protoplasts showed that the cassette blocks TGMV replication and severely impairs replication of heterologous begomoviruses.

8. Further experiments were carried out to test for transgene expression of a trans-dominant negative mutation and an RBR binding mutation and the effect of these mutations on viral resistance. Thus, a plant expression cassette carrying a variant of TGMV Rep (AL1) with a trans-dominant negative mutation (FQ118AA) and an RBR binding mutation (L148A) was transformed into *N. benthamiana*, and the resulting lines were tested for transgene expression and TGMV resistance.

Six T1 lines (out of 14 segregating 3:1 on kanamycin) expressed the transgene and showed reduced levels of viral DNA levels at 10 dpi (See Fig. 1A and B). Line 6 (L6) continued to display strong resistance at 17 and 23 dpi (Fig. 1C). T2 and T3 L6 plants were indistinguishable from uninfected, non-transgenic plants (Fig. 1D). L6 progeny of homozygous parents did not accumulate detectable levels of viral DNA (Fig. 1E), while progeny of heterozygous parents segregated for the trait.

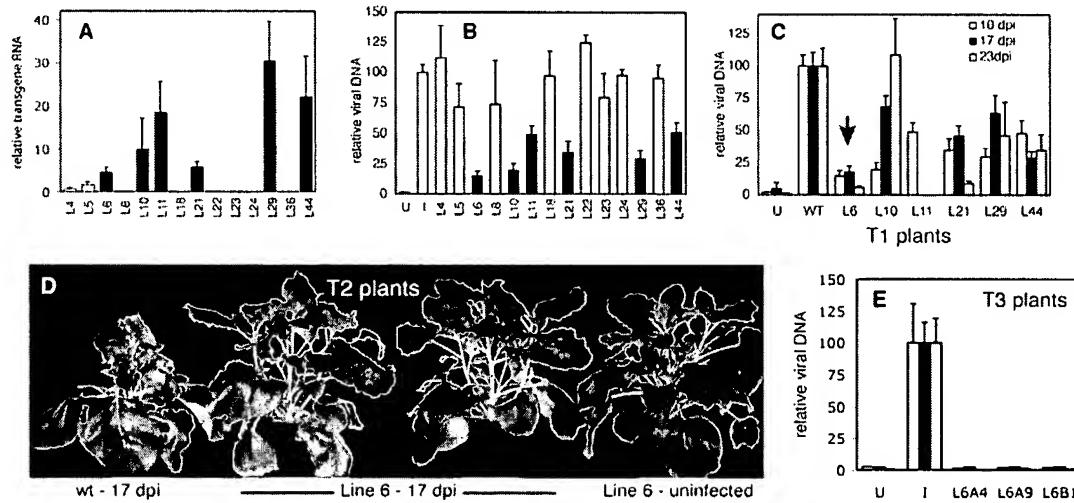


FIGURE 1. Plants co-expressing mutant TGMV Rep are immune to TGMV infection. A. Transgene RNA levels were analyzed by quantitative RT-PCR. B. Viral DNA levels in T1 lines at 10 dpi. The controls were uninfected (U) and infected (I) wild type plants. The lines marked by black bars were selected for further analysis. C. Viral DNA accumulation in T1 lines at 10, 17 and 23 dpi. Line 6 was selected for further analysis (red arrow). D. Resistant L6 T2 plants are asymptomatic and resemble uninfected wild type plants. E. Viral DNA levels in L6 T3 plants at 10, 17 and 23 dpi.

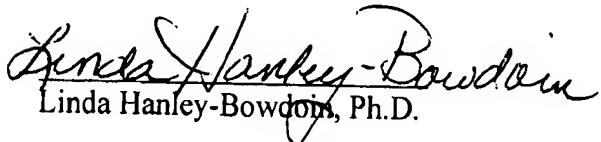
These results establish that the resistance trait is heritable and does not impact development. These properties are in sharp contrast to plants expressing Rep (AL1) proteins carrying an intact pRBR binding motif.

9. As mentioned in the specification, the phenotype of the L148 mutation is very similar to that of the KEE146 mutation, including symptoms in inoculated plants. (Specification, page 40, lines 10 through page 41, line 5, and Figure 9).

Further, the CbLCV L145 mutation (encoded by SEQ ID NO:109) is the functional equivalent of the TGMV L148 mutation as shown by both its location and by its Rb binding phenotype (Specification, page 42, lines 19-21, and Figure 11B, and Arguello-Astorga et al., J Virol. 78: 4817-4826 (2004), Fig. 5; copy enclosed). By analogy therefore, a scientist working in this field would reasonably expect that the presence of an L145 mutation, similar to an L148 mutation, would result in stable heritable resistance to geminivirus infection in transgenic plants, when used in combination with a transdominant negative mutation, as described in the specification (Specification, for example, at least, page 16, line 19 through page 17, line 2; and page 18, lines 7-11).

Thus, the data described in sections 6 and 7 above, in combination with what is described in the '850 application, show that transformation of a plant with an AL1 nucleic acid sequence encoding a trans-dominant mutation and an Rb binding mutation, such as L145 or L148, would be expected to result in stably expressed geminivirus resistance in plants.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.


Linda Hanley-Bowdoin, Ph.D.

5/4/07
Date